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The Formamidopyrimidine Derivative of 7-(2-Oxoethyl)-2'-deoxyguanosine

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Abstract

Vinyl chloride induces hepatic angiosarcomas, which are otherwise rare malignancies. The biochemical basis involves the formation of the epoxide, which reacts with DNA to give ~98% of the 7-(2-oxoethyl) adduct (**4**) of dGuo plus small amounts of the etheno derivatives of dGuo, dCyd, and dAdo. The carcinogenicity is generally ascribed to the etheno adducts, not **4**, because **4** has been shown to disappear from cells rapidly and to have negligible mutagenicity, which argues against its biological importance, whereas etheno adducts are both persistent and mutagenic. It has also been shown that apurinic sites derived from **4** are unlikely to be crucial lesions. A confounding factor with regard to the etheno hypothesis is that etheno adducts arise in unexposed cells by reactions of various lipid peroxidation products. The present study explores the possibility that a major contributor to the carcinogenicity of vinyl chloride may be formamidopyrimidine (FAPy) **12**, *N*-[2-amino-6-[(2-deoxy-β-*D*-erythro-pentofuranosyl)amino]-3,4-dihydro-4-oxo-5-pyrimidinyl]-*N*-(2-oxoethyl)-formamide, which can arise by ring opening of **4**, although its formation has not been observed until the present study. N7 adduct **4** undergoes deglycosylation to give 7-(2-oxoethyl)-Gua (**13**) in acid and imidazolium ring-opening to **12** in base. At pH 7.4, both processes occur with the formation of **12** representing ~10% of the product mixture. FAPy **12** spontaneously cyclizes to **22**, which upon mild acid treatment yields the deglycosylation product 2-amino-3,4,7,8-tetrahydro-7-hydroxy-4-oxopteridine-5(6*H*)-carbaldehyde (**14**). The structure of **14** has been established by NMR and mass spectroscopy and by independent synthesis. Reaction of the epoxide of crotonaldehyde with dGuo failed to give either **13** or **14**, indicating that both compounds are unique products of the reactions of dGuo with the epoxides of vinyl monomers. Although FAPy **12** was found to be unstable, carbinolamine **22** arising from cyclization of **12** may be an important contributor to the carcinogenicity of vinyl chloride.

Introduction

Vinyl chloride is an industrial chemical that is used widely for the preparation of polyvinyl chloride and copolymers. It is a carcinogen epidemiologically linked to hepatic angiosarcomas in humans and experimentally linked to the same tumors in rodents (1–4). Vinyl chloride is epoxidized by cytochrome P450 2E1 to chlorooxirane, which rapidly rearranges to chloroacetaldehyde (5). Chlorooxirane reacts extensively with DNA, whereas chloroacetaldehyde reacts mainly with proteins. Chlorooxirane alkylates dGuo at N1, N2, N3, and N7 to form the corresponding oxoethyl adducts **1–4** (Scheme 1). The N1 and N3

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Supporting Information Available: Gradient protocols for HPLC separations; ¹H spectra of **4**, **14**, **16–18**, and **21**; NOESY spectrum of **14**, ¹³C spectrum of **14**; UV spectra of **14** and **21**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

adducts cyclize to give 1,*N*²- and *N*²,3-etheno-dGuo (**5** and **7**), respectively; the *N*² adduct cyclizes to form 8-hydroxy-5,6,7,8-tetrahydropyrimido[1,2-*a*]purin-10(3*H*)-one (**6**). Reaction also occurs with dCyd at N3 and with dAdo at N1 to give oxoethyl derivatives **8** and **9**, respectively; these cyclize to etheno derivatives **10** and **11**. In the reaction of chlorooxirane with DNA, N7 adduct **4** is by far the major adduct, representing ~98% of the product mixture (6). Nevertheless, the etheno adducts are generally considered to be biologically more important because **4** has been reported to be nonmutagenic (7) and short-lived (8), whereas the etheno species are miscoding *in vitro* (9–12) and both mutagenic and highly persistent *in vivo* (13).

A confounding factor has been that the etheno adducts are present in the cells of laboratory animals and humans not exposed to vinyl chloride or other vinyl monomers that might be capable of forming etheno adducts (14). The endogenous etheno derivatives are believed to arise from the epoxides of 4-hydroxy-2-nonenal and other enals formed as oxidation products of unsaturated lipids (15) and chemical studies support this notion (16–18). Thus, a central question is why low levels of exposure to vinyl chloride would generate sufficiently high concentrations of etheno adducts to create a substantial risk of inducing malignancies when significant background levels of these adducts are already present in normal cells. One needs to consider the possibility that some other, as yet unexamined, adduct of chlorooxirane is the primary cause of angiosarcomas resulting from vinyl chloride exposure, with an important qualification being that the adduct should not also be formed by reactions of lipid peroxidation products and thus not present in unexposed cells.

The N7 adducts of dGuo are cationic and, as a consequence, unstable. Studies of other N7 adducts in DNA have shown that they undergo (a) nonenzymic deglycosylation to 7-substituted Gua and leave apurinic sites in the DNA (19–21) and (b) hydrolysis of the imidazole ring to create formamidopyrimidine (FAPy¹) derivatives (22–24). Depurination is an acid-catalyzed reaction (25, 26); FAPy formation requires a stoichiometric equivalent of hydroxide ion (24, 27, 28). FAPy lesions derived from 7-alkyl dGuo adducts have been characterized in biological samples and prepared by chemical routes (29–33). They have been found to exist as complex mixtures of stereoisomers due to hindered rotation around the C5-N⁵ bond leading to atropisomers and slow reorientation of the planar formamide creating geometrical isomers (29, 34). An additional problem involves opening and reclosure of the deoxyribose ring leading to a mixture of α - and β -furanosides in DNA (29). With the free deoxyribosides, the furanosides can also isomerize to pyranosides (29, 34, 35). Pyranose formation cannot occur in DNA and is therefore not relevant to the replication of FAPy lesions (33). However, the detection of DNA adducts usually involves the digestion of DNA to the individual nucleosides; therefore, the isomerization of the FAPy furanose to the pyranose is a factor in analysis. In some cases the various isomeric forms are separable but still able to interconvert causing the nucleosides to have poor chromatographic behavior and complex NMR spectra. Consequently, there is a reasonable possibility that formamidopyrimidine **12** (oxoethyl-FAPy) arises from **4** but has escaped detection in biological samples. In addition, **12** has never been synthesized; the lack of an authentic sample complicates detection of **4**. Thus, the failure of investigators ever to observe **12** in reactions of chlorooxirane with DNA or in cells exposed to vinyl chloride might reflect these problems rather than the absence of **12**.

In this article, we examine the hypothesis that the oxoethyl-FAPy lesion derived from the N7 adduct of dGuo (**4**) is formed in significant quantities at physiological pH by reaction of the epoxide of vinyl chloride with dGuo and therefore might be an important contributor to the carcinogenicity of vinyl chloride. We find that cationic adduct **4** undergoes both

¹Abbreviations: FAPy, formamidopyrimidine; ESI, electrospray ionization; RE, relative energy.

depurination to give 7-oxoethyl-Gua (**13**) and ring-opening to form FAPy nucleoside **12** with FAPy formation at physiological pH representing ~10% of the product mixture whereas it has been reported that the etheno adducts are no more than 2% (**6**).

Materials and Methods

All commercial chemicals were of the highest quality commercially available and used without further purification. Chlorooxirane and dimethyldioxirane were prepared by published procedures (36, 37).

NMR Spectra

Unless otherwise noted, ^1H NMR spectra were recorded at 400 or 500 MHz in D_2O , acetone- d_6 or DMSO- d_6 ; ^{13}C NMR spectra were recorded at 125 MHz. The two-dimensional nuclear Overhauser/chemical exchange spectroscopy experiments (NOESY) were performed on a 500 MHz spectrometer with the water peak suppressed by presaturation. A total of 4096 scans were collected using a spectral width of 5000 Hz. The acquisition, preacquisition delay, and mixing times were 204 ms, 2 s, and 600 ms, respectively.

The ^1H spectrum of N7 adduct **4** was obtained at 600 MHz using a Bruker LC-NMR accessory operated in the stop-flow mode. The sample was eluted from a C-18 reverse phase column (Bruker Install, 125×4 mm, flow rate 1 mL/min) and sent directly to a 120 μL flow cell (Bruker Cryofit) in a 5 mm cryogenically cooled NMR probe (TCI). The HPLC solvent system comprised 0.1 M ammonium formate buffer (pH 7.0) in D_2O and acetonitrile and employed a gradient from 3% to 97% acetonitrile. Effluent composition was monitored at 254 nm. ^1H spectra were acquired using the WET solvent suppression pulse sequence to reduce signals associated with residual water and acetonitrile. For 1D ^1H NMR, typical experimental conditions included 32K data points, 20 ppm sweep width, a recycle delay of 1.5 s and 32–256 scans depending on sample concentration. For 2D ^1H - ^1H COSY, experimental conditions included 2048×256 data matrix, 13 ppm sweep width, recycle delay of 1.5 and 4 scans per increment. The data were processed using squared sinebell window function, symmetrized, and displayed in magnitude mode (38).

Chromatography

HPLC analysis was carried out on a gradient instrument (Beckman Instruments: pump module 125, photodiode array detector module 168, and System Gold software). For monitoring reactions, C-18 reverse phase columns (YMC ODS-AQ, 250×4.6 mm, flow rate 1.5 mL/min and Phenomenex Gemini-C18, 250×4.6 mm, flow rate 1.5 mL/min) were used. Sample purifications were carried out using larger C-18 reverse phase columns either by HPLC (Phenomenex Gemini-C18 column, 250×10 mm, flow rate 5 mL/min and YMC ODS-AQ, 250×10 mm, flow rate 5 mL/min) or by preparative chromatography (Biotage SP1 (Charlottesville, VA) with a C18-HS-(12+M) column, flow rate 12 mL/min). The details of solvent gradients are provided in the Supporting Information. Normal phase HPLC analysis was performed with a Phenomenex Luna 5 μm Silica column ($150 \text{ mm} \times 2 \text{ mm}$, flow rate 0.60 mL/min). In all cases, effluent compositions were monitored at 254 nm.

Mass Spectrometry

FAB mass spectra (low and high resolution) were obtained at the Mass Spectrometry Facility at the University of Notre Dame, Notre Dame, IN. LC-ESI/MS was performed on a DecaXP ion trap instrument (ThermoFinnigan, San Jose, CA) using an Agilent 1100 A pump system (Agilent, Foster City, CA) and operated in the positive ion mode unless otherwise noted. Electrospray spectra were obtained with a Finnigan LTQ mass

spectrometer (ThermoElectron) in the Vanderbilt Mass Spectrometry Facility. Detection and quantification of **14** were carried out on a Phenomenex Luna 5 μm silica (150 mm $\times$ 2 mm) column, flow rate of 0.35 mL/min using gradient G, (see Supporting Information); **14** eluted at 7.27 min. Samples were injected using an autosampler. ESI conditions: source voltage 4 kV, N_2 sheath gas setting 64 units, N_2 auxiliary sweep gas setting 11 units, capillary voltage 3 V, capillary temperature 300 $^\circ\text{C}$, tube lens offset 0 V. A method consisting of three scan events was used: (1) full scan, 2 microscans, ion accumulation time 200 ms, m/z [100.00–500.00]; (2) selected reaction monitoring: 1 microscan, spectral width 2, ion accumulation time 50 ms, MS m/z 212.10 at 25 [165.50–166.50, 183.50–184.50]; (3) MS³ 1 microscan, spectral width 2, ion accumulation time 200 ms, m/z 212.10 $\rightarrow$ m/z 166 at 35 [100–170].

Acetoxoxirane

Vinyl acetate (1.04 mL, 17.0 mmol) was added dropwise to an acetone solution of dimethyldioxirane (375 mL, 0.05 M) at -78 $^\circ\text{C}$. The mixture was allowed to warm to room temperature. After 1 h, the mixture was concentrated under vacuum (70–80 Torr). Distillation was stopped after the volume had been reduced by $\sim 30\%$. The remaining solution was dried with anhydrous K_2CO_3 for 15 min at 0 $^\circ\text{C}$, filtered and distilled (80 $^\circ\text{C}$, 70–80 Torr) to give acetoxoxirane (1.8 g, 78%). ^1H NMR (acetone- d_6): δ 5.36 (dd, 1H, $J = 2.4$ Hz, $J = 1.2$ Hz), 2.72 (dd, 1H, $J = 2.4$ Hz, $J = 4.4$ Hz), 2.69 (dd, 1H, $J = 1.2$ Hz, $J = 4.4$ Hz), 1.94 (s, 3H).

Reaction of Acetoxoxirane with dGuo in Phosphate Buffers

dGuo·H₂O (0.485 mg, 0.0017 mmol) in degassed phosphate buffers (100 mM, pH 7, pH 8 or pH 9) (200 μL) was treated with acetoxoxirane (0.26 mmol, 24 μL) at room temperature. The progress of the reaction was monitored by HPLC (YMC ODS-AQ column, gradient F) and the LC-ESI/ MS/MS method for the detection of oxoethyl-FAPy.

Reaction of dGuo and Acetoxoxirane in DMSO

dGuo·H₂O (0.5 mg, 0.0016 mmol) in anhydrous, degassed DMSO (100 μL) was treated with acetoxoxirane (0.26 mmol, 24 μL) at room temperature. Aliquots (25 μL) were withdrawn at 15, 30, and 60 min and treated with 0.5 M NaOH (200 μL) for 3 min. To these solutions 6 M HCl (<10 μL) was added to lower the pH to 2–3; the mixtures were allowed to stand at room temperature for 1.5 h. The mixtures were spiked with measured quantities (0.302 μg) of d_3 -**14** as an internal standard and analyzed by LC-ESI/MS/MS (10 μL samples). Quantitation involved comparison of the peak for signals at m/z 212 in the full spectra with the respective signals derived from d_3 -**14**, which appeared at m/z 215.

Synthesis of 7-(2-Oxoethyl)-2'-deoxyguanosinium Ion (**4**)

dGuo·H₂O was treated with acetoxoxirane (10 eq) for 3 h in glacial acetic acid. Oxoethyl-dGuo salt **4** was isolated by precipitation with diethyl ether. HPLC analysis of the product mixture showed a peak at 7.8 min for **4** and a peak at 11 min for unreacted dGuo. Attempts to achieve complete alkylation of dGuo using larger excesses of acetoxoxirane led to multiple alkylation products, which complicated purification of **4**. Salt **4** was purified by reverse phase HPLC (Phenomenex Gemini-C18 column, gradient F) and the solution was immediately frozen at -78 $^\circ\text{C}$. The ESI/MS spectrum, obtained by loop injection, gave molecular ions at m/z 310 and 328 for **4** and its hydrate, respectively. In addition, fragment ions were observed at m/z 194, 177 and 152, with the 194 signal being the base peak in the spectrum. Fragmentation of the molecular ion m/z 310 at 10% RE gave a product ion of m/z 194, which is assigned as loss of deoxyribose. Further fragmentation of m/z 194 at 20% RE gave fragment ions at m/z 177, 166 and 152. Molecular ion peak with m/z 328 at 10% RE gave fragment ion m/z 212 ion, which at 20% RE gave ions m/z 194 and 152.

The ^1H NMR spectrum of **4** was obtained by LC-NMR in the stop-flow mode (see Materials and Methods). ^1H NMR (ammonium formate/ D_2O buffer, pH 7 and acetonitrile): δ 6.27 (t, 1H, $J = 6$ Hz, H-1'), 5.30 (t, 1H, $J = 6$ Hz, N- $\text{CH}_2\text{CH}(\text{OH})_2$), 4.45 (m, 1H, H-3'), 4.39 (d, 2H, $J = 6$ Hz, N- $\text{CH}_2\text{CH}(\text{OH})_2$), 4.05 (m, 1H, H-4'), 3.73–3.71 (m, 1H, $J_1 = 12$ Hz, $J_2 = 6$ Hz, H-5'), 3.66–3.63 (m, 1H, $J_1 = 12$ Hz, $J_2 = 6$ Hz, H-5'), 2.66–2.61 (m, 1H, H-2'), 2.55–2.50 (m, 1H, H-2'). The H-8 signal was not observed due to exchange with D_2O . Coupling between the methylene and methine signals of the dihydroxy-ethyl group was confirmed by a COSY spectrum.

The structure of **4** was established by deglycosylation to form **13** by pH 1.0 treatment for 2 h at room temperature. The purity of **4** was established by treatment with 0.1 M NaOH for 15 min followed by acid hydrolysis (0.5 M aqueous HCl) for 1 h at 90 °C to give **21** after neutralization and contact with air. HPLC analysis showed ~2% contamination of **4** by **13**. 2-Aminobenzyl alcohol was employed as the internal standard for this analysis.

The chloride salt was prepared similarly by treatment of dGuo with chlorooxirane in glacial acetic acid.

Reactivity of 7-(2-Oxoethyl)-2'-deoxyguanosinium Salt (**4**)

The purified salt of **4** was incubated in buffers at pH values ranging from 6.5 to 10.0 at 37 °C to determine partitioning between deglycosylation and FAPy formation. The reactions were carried out with **4** prepared both from acetoxoxirane and chlorooxirane. Aliquots were withdrawn after 24 h and quantitated by HPLC (Phenomenex Gemini-C18 column, gradient F) to estimate the fraction of **13** being formed. In parallel, a second set of aliquots were withdrawn and treated with aqueous HCl for 1 h at 90 °C and compound **21** was analyzed using gradient F (Phenomenex Gemini-C18 column). For quantitation of **13** and **21**, known amounts of 2-aminobenzyl alcohol were added as an internal standard.

N-[2-Amino-6-[(2-deoxy- β -D-erythro-pentofuranosyl)amino]-3,4-dihydro-4-oxo-5-pyrimidinyl]-*N*-(2-propenyl)-formamide (**16**)

dGuo- H_2O (300 mg, 1.05 mmol) was dissolved in degassed DMSO (10 mL). Allyl bromide (1.94 mL, 22.40 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 3 h. Excess allyl bromide was removed *in vacuo* and the residue was treated with NaOH (30 mL, 1 M) for 1 h. The reaction was monitored by HPLC (YMC ODS-AQ column, gradient A) and purified by chromatography (Biotage SP1, gradient D) to afford **16** (150 mg, 41%). ^1H NMR ($\text{DMSO}-d_6$) mixture of isomers: δ 10.69 (broad s, 1H), 7.76–7.69 (multiple s, 1H, CHO), 7.06 (d, 1H, NH, $J = 7.6$ Hz), 6.64 (broad s, 2H, NH_2), 5.77–5.74 (m, 1H, =CH-), 5.42–5.35 (m, 1H, H-1'), 5.14–4.97 (m, 3H, OH-3', = CH_2), 4.70–4.43 (m, 1H, OH-5'), 4.10–3.85 (m, 3H, H-3', CH_2N), 3.78–3.65 (m, 1H, H-4'), 3.67–3.24 (m, 2H, H-5'), 1.93–1.71 (m, 2H, H-2'). HRMS (FAB+) m/z calcd for $\text{C}_{13}\text{H}_{20}\text{N}_5\text{O}_5$ [$\text{M} + \text{H}$] $^+$ 326.1464, found 326.1449.

N-(2,6-Diamino-3,4-dihydro-4-oxo-5-pyrimidinyl)-*N*-(2-propenyl)-formamide (**17**)

Compound **16** (130 mg, 0.39 mmol) was heated in 5 mL of 1 M HCl for 2 h at 60 °C. The reaction was monitored by HPLC (YMC ODS-AQ column, gradient A) and purified by chromatography (Biotage SP1, gradient D) to afford **17** (75 mg, 92%). ^1H NMR ($\text{DMSO}-d_6$) first isomer: δ 11.50 (broad s, 1H), 8.34 (s, 1H, CHO), 6.36–6.30 (broad s, 2H, NH_2), 5.81–5.71 (m, 1H, =CH-, $J = 8$ Hz), 5.11–5.03 (m, 2H, = CH_2), 4.03–3.97 (m, 1H, CH_2N); second isomer: δ 11.35 (broad s, 1H), 7.74 (s, 1H, CHO), 6.14–6.00 (broad s, 2H, NH_2), 5.70–5.68 (m, 1H, =CH-, $J = 8$ Hz), 4.99–4.95 (m, 2H, = CH_2), 3.90–3.78 (m, 2H, CH_2N). HRMS (FAB+) m/z calcd for $\text{C}_8\text{H}_{12}\text{N}_5\text{O}_2$ [$\text{M} + \text{H}$] $^+$ 210.0991, found 210.0997.

***N*-(2,6-Diamino-3,4-dihydro-4-oxo-5-pyrimidinyl)-*N*-(2,3-dihydroxypropyl)-formamide (18)**

A solution of **17** (70 mg, 0.33 mmol) in water (0.3 mL) was added to a mixture of water (1 mL), acetone (0.5 mL), *N*-methylmorpholine-*N*-oxide (46.39 mg, 0.39 mmol) and OsO₄ (~1 mg). The reaction mixture was stirred overnight at room temperature followed by evaporation of the solvents. Progress of the reaction was monitored by HPLC (YMC ODS-AQ column, gradient B). The product mixture was purified by chromatography (Biotage SP1, gradient D) to afford **18** (69 mg, 85%). ¹H NMR (DMSO-*d*₆) mixture of isomers: δ 12.00 (broad s, 1H), 8.07–7.74 (s, 1H, CHO), 6.70–6.50 (broad s, 1H, NH₂), 6.43–6.20 (broad s, 1H, NH₂), 4.02–3.99 (m, 1H, CH₂OH), 3.77–3.75 (m, 1H, CH₂OH), 3.53–3.48 (m, 1H, CHOH), 3.35–3.25 (m, 2H, CH₂N). HRMS (FAB+) *m/z* calcd for C₈H₁₄N₅O₄ [M + H]⁺ 244.1046, found 244.1058.

2-Amino-3,4,7,8-tetrahydro-7-hydroxy-4-oxopteridine-5(6*H*)-carbaldehyde (14)

Compound **18** (60 mg, 0.25 mmol), dissolved in water (1 mL), was treated with aqueous NaIO₄ solution (16 mL, 20 mM in 0.05 M phosphate buffer, pH 7) for 45 min at room temperature. The reaction was monitored and purified by HPLC using a Phenomenex Gemini-C18 column (gradient C; solvent system A consisted of 0.1 M aqueous ammonium formate buffer) to afford, after being lyophilized several times to remove ammonium formate, **14** (42 mg, 79%) as a mixture of geometrical isomers. ¹H NMR (500 MHz, D₂O, 10% CCl₃COOD) isomer **14a**: δ 7.89 (s, 1H, CHO), 5.23 (broad t, 1H, CHOH), 3.76 (dd, 1H, CH₂, *J*₁ = 1.8 Hz, *J*₂ = 13.4 Hz), 3.11 (dd, 1H, CH₂, *J*₁ = 1.8 Hz, *J*₂ = 13.4 Hz); isomer **14b**: δ 8.56 (s, 1H, CHO), 5.20 (broad t, 1H, CHOH), 4.42 (dd, 1H, CH₂, *J*₁ = 1.8 Hz, *J*₂ = 18.0 Hz), 2.73 (dd, 1H, CH₂, *J*₁ = 1.8 Hz, *J*₂ = 18.0 Hz). ¹³C NMR (125 MHz, D₂O, 10% CCl₃COOD) isomer **14a**: δ 163.55, 158.13, 154.25, 153.45, 91.72, 72.70, 42.32; isomer **14b**: δ 165.42, 158.79, 155.25, 153.84, 93.04, 72.70, 48.10. HRMS (FAB+) *m/z* calcd for C₇H₁₀N₅O₃ [M + H]⁺ 212.0784, found 212.0793. UV (H₂O) λ_{max} 290 nm.

2-Amino-5,8-dihydro-4(3*H*)-pteridinone (20)

Carbinola-mine **14** (5 mg, 0.023 mmol) was dissolved in D₂O (0.5 mL) containing CCl₃COOD (0.05 mL). The solution was heated in an NMR tube for 2 d at 60 °C to give **20**. The reaction was monitored by HPLC (Phenomenex Gemini-C18 column, gradient C). ¹H NMR (500 MHz, D₂O, 10% CCl₃COOD): δ 8.85 (d, 1H, *J* = 2.3 Hz), 8.78 (d, 1H, *J* = 2.3 Hz); ¹³C NMR (125 MHz, D₂O, 10% CCl₃COOD): δ 165.75, 160.00, 150.09, 147.83, 144.32, 128.22. HRMS (FAB+) *m/z* calcd for C₆H₈N₅O [M ± H]⁺ 166.0729, found 166.0701.

2-Aminopteridone (2-Amino-4-hydroxypyrimido[4,5-*b*]pyra-zine, 21)

Compound **21** was synthesized as previously described (39). ¹H NMR (500 MHz, D₂O, 10% CCl₃COOD): δ 7.48 (d, 1H, *J* = 2.2 Hz), 7.39 (d, 1H, *J* = 2.2 Hz); ¹³C NMR (125 MHz, D₂O, 10% CCl₃COOD): δ 159.83, 151.85, 150.99, 147.65, 143.90, 127.90. HRMS (FAB+) *m/z* calcd for C₆H₆N₅O [M + H]⁺ 164.0572, found 164.0565. UV (H₂O) λ_{max} 270, 343 nm.

Preparation of *d*3-oxoethyl-FAPy (*d*₃-14)

*d*₃-Oxoethyl-FAPy was synthesized according to Scheme 2 using *d*₅-allyl bromide. The structure was confirmed by ¹H NMR, MS and UV. ESI-MS analysis showed the anticipated molecular ion peak at *m/z* 215.

Failure of 2,3-Epoxybutenal to Form N7 Adduct 4

dGuo was treated with 2,3-epoxybutenal in three solvents: (a) pH 7.0 phosphate buffer, (b) dry, degassed DMSO, and (c) glacial acetic acid following the procedures employed for the reactions of dGuo with acetoxy- and chlorooxirane. To take into account the lower reactivity

of the epoxyaldehyde, reaction times were extended to 48 h, 12 h and 10 h, respectively. In each case, samples were treated with 0.5 M HCl to look for formation of **13**. Additional samples were treated with 0.1-1.0 M NaOH and then assayed for FAPy formation by conversion to **21**. In no case was **13** or **21** detected.

Results

Chlorooxirane was prepared in pure form by photochemical chlorination of ethylene oxide with chlorine as previously reported (36). It can also be synthesized using t-butyl hypochlorite as the chlorination agent (40, 41), but product prepared by that method cannot readily be freed of t-butanol and HCl. Acetoxyoixirane was conveniently prepared by epoxidation of vinyl acetate with dimethyldioxirane. The analogous epoxidation of vinyl chloride occurs too slowly to be synthetically useful and the epoxide codistills with acetone. Acetoxyoixirane is more stable than chlorooxirane but somewhat less reactive with dGuo. Hydrolysis of acetoxyoixirane by adventitious moisture leads to glycolaldehyde, which does not form dGuo adducts whereas chloroacetaldehyde derived from chlorooxirane will. The convenience of working with acetoxyoixirane led us to explore its reactions with dGuo first and subsequently reproduce these findings with chlorooxirane.

The reaction of acetoxyoixirane with dGuo in aqueous buffers gave mainly 7-oxoethyl-Gua (**13**); minor amounts of glyoxal-dGuo, 1,*N*²-etheno-dGuo and *N*²,3-etheno-dGuo were also isolated. In no case was it possible to isolate *N*7 adduct **4** which went on to **13** too rapidly. In a reaction carried out in DMSO, salt **4** was undoubtedly formed but again underwent deglycosylation. Products were identified by coinjection with authentic standards. Formation of the glyoxal adduct of dGuo was unexpected. Glyoxal could arise either by oxidation of glycolaldehyde or acetoxyoixirane. In later studies, formation of glyoxal and its dGuo adduct was suppressed by carrying out the reactions using degassed solvent under an inert atmosphere.

To confirm that deglycosylation was occurring in the DMSO solution rather than during HPLC analysis, dGuo and acetoxyoixirane were incubated in DMSO for 20 min, the DMSO was evaporated in vacuo, and phosphate buffers ranging from pH 7.0 to 9.0 were added to promote conversion of **4** to FAPy **12**. In all cases, HPLC analysis showed 7-oxoethyl-Gua (**13**) to be the major product. No peak was observed that could be ascribed to FAPy nucleoside **12**.

As discussed in the introduction, we recognized the possibility that FAPy nucleoside **12** might have complex chromatographic behavior such that the product could be overlooked. The mixture of stereoisomers could be simplified somewhat by analyzing the base rather than the nucleoside, although the potential remained for the product to still be a mixture of C5-*N*⁵ atropisomers and formamide geometrical isomers. Product mixtures potentially containing **12** were treated for 2 h at pH 1.0 and room temperature to bring about deglycosylation. LC-ESI/MS/MS analysis of these acid hydrolysates gave a chromatographic peak having a molecular ion at *m/z* 212 which is consistent with [M + H]⁺ for FAPy base **19**.

Only minute amounts of the putative FAPy base **19** were being formed. Consequently, an independent synthesis was developed to confirm its structure. The approach involved preparation of allyl-FAPy base **17** by alkylation of dGuo with allyl bromide (Scheme 2). Cationic adduct **15** was immediately treated with sodium hydroxide to give allyl-FAPy nucleoside **16** in 41% overall yield after purification. Purification was complicated by the fact that **16** was a mixture of stereoisomers and had poor chromatographic properties. Acid-catalyzed de-glycosylation gave FAPy base **17** in high yield; the HPLC properties of **17**

were somewhat better than those of **16**. Dihydroxylation of **17** using OsO₄ and *N*-methylmorpholine oxide gave dihydroxypropyl-FAPy **18** in 85% yield. Oxidative cleavage of **18** with NaIO₄ at neutral pH gave **19** in 79% yield.

HPLC analysis carried out immediately after the periodate oxidation gave a broad peak, *t_R* ~5 min, for **19** when analyzed by reverse phase HPLC. Solubility and stability constraints precluded obtaining NMR spectra. On standing, the material underwent a spontaneous transformation to give a pair of peaks, *t_R* 2.89 and 3.57 min, along with a broad peak between them. This change occurred over a period of 1 h but was more rapid if a trace of acid was added. The sharp peaks were collected individually at -78 °C. Reanalysis of the two fractions by HPLC again gave a chromatogram showing two peaks with a shoulder between for each of them (Figure 1A), indicating that two species were interconverting on the time scale of the separation. When a normal phase chromatographic system was used a single, slightly broadened peak was obtained (Figure 1B). Subsequently, these components were collected by reverse phase HPLC as a single fraction. The two peaks are assigned as geometrical isomers of carbinolamine **14** (Scheme 3) on the basis of spectra and chemical transformations. The mass spectrum acquired by LC-ESI/MS/MS, showed a parent ion [M + H]⁺ at *m/z* 212 with loss of CO to form a *m/z* 184 ion followed by further fragmentation to form lighter ions.

NMR studies were carried out on **14** in D₂O; trichloroacetic acid was added to obtain adequate solubility. No aldehyde signal in the 9.0–10.0 ppm range was observed in the ¹H NMR spectrum, indicating that oxoethyl-FAPy **19** was not present in any detectable amount. The two forms of **14** are designated as rotamers **14a** and **14b**. The formamide protons of **14a** and **14b** were observed at 7.89 and 8.56 ppm, respectively. In both species the methine proton appeared as a broad triplet at 5.17 ppm. The methylene signals of **14a** appeared at 3.11 and 3.76 ppm, whereas those of **14b** appeared at 2.73 and 4.42 ppm. It is noteworthy that the difference in chemical shifts for the methylene protons of **14a** is much smaller than the difference for **14b**. Strong geminal coupling was observed between the methylene protons (13.4 Hz in **14a** and 18 Hz in **14b**) and weak vicinal coupling (1.8 Hz) to the methine protons; this relationship was confirmed by a COSY spectrum. The NOESY spectrum of **14a** showed correlation of the formyl proton with one of the methylene protons (3.11 ppm) allowing this isomer to be assigned as having a *Z* conformation with the formyl proton proximal to the methylene group. No correlation was observed to the other methylene proton. With **14b**, no correlation was observed with either of the methylene protons, which is consistent with an *E* configuration for the formamide. The NOESY spectrum showed an exchange cross-peak between the formyl proton signals arising from reorientation of the formyl group on the time scale of the NMR experiment. The ¹³C chemical shifts were correlated with the proton spectrum via an HSQC spectrum. The spectra of **14a** and **14b** were very similar with the exception that the methylene carbon of **14a** was 5.7 ppm farther downfield than the methylene carbon of **14b** (42.3 versus 48.1 ppm). This is consistent with the methylene carbon of **14b** lying in the shielding cone of the carbonyl group.

Carbinolamine **14** and the hydrate of 7-oxoethyl-Gua (**13**) have the same molecular weight. The ESI-MS/MS spectrum of **13** contained species corresponding to **13** (*m/z* 194) and its hydrated form (*m/z* 212). At 25% RE, the *m/z* 194 ion lost the oxoethyl group to give Gua, *m/z* 152. Compound **14** has a molecular ion at *m/z* 212, which loses CO at 15% RE to give product ion *m/z* 184. At 20% RE, the *m/z* 212 gave the *m/z* 184 ion plus a product ion at *m/z* 166 reflecting loss of water from the *m/z* 184 ion. At 30% RE, the *m/z* 184 ion fragmented to form product ion *m/z* 166. The *m/z* 212 → 184 transition would not distinguish **14** from the hydrate of **13** but at 30% RE it gave only the *m/z* 166 product ion. MS³ fragmentation was carried on the *m/z* 166 product ion at 35% RE. This led to the formation of a major product ion *m/z* 149 due to loss of ammonia, plus less intense product ions at *m/z* 138 and *m/z* 124,

assigned as losses of CO and NC-NH₂, respectively. Based on these MS fragmentations, an LC-ESI/MS/MS method was developed for detection of carbinolamine **14** consisting of a full scan followed by fragmentation of the *m/z* 212 molecular ion at 25% RE to give product ions *m/z* 166 and *m/z* 184, then in a third event *m/z* 212 was fragmented at 30% RE to give only *m/z* 166, which was further fragmented at 35% RE. Possible structures for the product ions derived from **14** are shown in Scheme 4.

The D₂O/CCl₃COOD solution of **14** that had been used for NMR measurements was heated for 2 d at 60 °C. HPLC analysis revealed the formation of a single product (*t_R* 5.18 min) with λ_{max} 270 and 343 nm. ¹H and ¹³C NMR data (D₂O/CCl₃COOD) suggested the compound was the protonated form of dihydropteridinone **20**. Compound **20** underwent facile air oxidation under neutral conditions to 2-aminopteridone (**21**) (42–45), which was identified by comparison with an authentic sample prepared by a published procedure (39). Compounds **20** and **21** are readily distinguished by their NMR spectra. HPLC and LC-ESI/MS/MS of **20** under neutral conditions showed only **21**.

With an authentic sample of carbinolamine **14** in hand, the reaction of acetoxoxirane with dGuo was repeated in anhydrous, oxygen-free DMSO. Work-up involved treatment with 0.5 M NaOH to convert cation **4** to FAPy nucleoside **12**, followed by treatment under acidic conditions (pH 1, 2 h, room temperature) to bring about cyclization to the carbinolamine and deglycosylation to give **14**. The maximum yield (4.5%) of **14** was observed after a 1 h reaction of acetoxoxirane with dGuo. Longer reaction times gave lower yields, possibly because acetoxoxirane was undergoing decomposition to form acetic acid which in turn caused deglycosylation of **4**; **13** was the primary product in all of these reactions.

A shortcoming of the above approach was that decomposition of the epoxide, deglycosylation of **4**, and other reactions occurred too rapidly to permit isolation of **4**. As a consequence, a clear distinction could not be made between failure of **4** to be formed and failure of **4** to form FAPy. To distinguish these processes, a method was needed by which **4** could be prepared in pure form so that the partitioning of **4** between FAPy formation and deglycosylation could be systematically studied.

A number of examples of the reactions of dGuo with electrophiles have been reported in which glacial acetic acid was used as the solvent including the reaction of chlorooxirane with dGuo (46–48). dGuo has excellent solubility in acetic acid. In spite of the acidity, depurination is not a serious problem if care is taken to keep the reaction mixtures anhydrous. Treatment of dGuo with acetoxoxirane in glacial acetic acid gave **4** with little concomitant deglycosylation. The salt was isolated by precipitation with diethyl ether. HPLC analysis of the crude product showed a peak for **4** at 7.8 min plus unreacted dGuo at 11 min. The product was purified by reverse phase HPLC at pH 7.0 and the solution of **4** was immediately stored at –78 °C. HPLC analysis of the purified material showed that **4** was relatively pure; the purity of **4** was confirmed by treatment with 0.1 M NaOH for 15 min followed by acid hydrolysis (0.5 M HCl) for 1 h at 90 °C to give **21**. Less than 3% of 7-oxoethyl-Gua (**13**) was observed; this is likely to be the maximum extent to which the deglycosylation product contaminated the chromatographed sample of **4**. Dilute solutions of **4** were relatively stable in ammonium formate buffer at pH 7 and room temperature but slowly deglycosylated to form **13**.

The structure of **4** was established by a) hydrolysis (16 h, pH 6.5, 37 °C) to form **13**, b) its mass spectrum which gave a weak but reproducible parent ion at *m/z* 310 and a strong fragment ion at *m/z* 194 for loss of deoxyribose, and c) its NMR spectrum. NMR analysis of **4** was initially attempted in deuteriated phosphate buffer at pH 7 and in deuteriated DMSO but **4** decomposed too rapidly to obtain useful spectra. Decomposition involved

deglycosylation to some extent but mainly self-condensation; the latter was a problem because of the high concentrations present during concentration of HPLC fractions and required to obtain NMR spectra. Attempts to achieve higher conversions of dGuo to **4** by using larger excesses of acetoxoxirane were unsuccessful due to addition of multiple acetoxoxirane moieties. These oligomeric byproducts complicated the purification of **4**; structural identification of them was not undertaken. The problem was resolved by stop-flow LC-NMR. A 600 MHz spectrometer equipped with a cryoprobe provided sufficient sensitivity that concentration of HPLC fractions was not required and self-condensation of **4** was minimal. The spectra confirmed the structure of **4**; and showed that the aldehyde was hydrated, i.e., **4a**, in the aqueous solvent (Scheme 5). The methine proton appeared at δ 4.5. The methylene protons (δ 5.3) were isochronous which argues against cyclic hemiacetal **4b** where nonequivalence would be greater.

Compound **4** was most stable at pH 8, the half-life for disappearance was about 5 h. As the pH was either raised or lowered, the rate of disappearance of **4** increased. The fate of **4** in buffers having pH values ranging from 6.5 to 10.0 was examined. In this study, reactions were allowed to go to completion (24 h, 37 °C) and the final product ratios analyzed. 7-Oxoethyl-Gua (**13**) was quantitated directly by HPLC. The instability of **12** precluded direct quantitation. Carbinolamine **22** resulting from cyclization of **12** and its deglycosylation product (**14**) were unattractive for quantitation due to their existence in multiple stereoisomeric forms. Therefore, pteridone **21** was chosen for quantitation of **12** (Scheme 6). Aliquots of the reaction mixtures were treated with dilute HCl for 1 h at 90 °C bring about formation of **14**, which lost the formyl group and dehydrated to give dihydropteridone **20**. After neutralization, exposure of **20** to air gave pteridone **21**, which was analyzed by HPLC. To aid in the quantitation of **13** and **21**, an internal standard, 2-aminobenzyl alcohol, was added. Calibration curves were generated and control experiments established that the internal standard was stable under all conditions employed in these studies.

HPLC analysis showed that the partitioning of **4** between the two pathways was pH dependent such that ring-opening was the exclusive pathway observed at pH 10.0 and depurination the dominant pathway at pH 6.5 (Table 1). At pH 7.4, **21** was 10% of the combined yield of **13** and **21**. The combined yields of the two products only approached 100% at the extremes of pH. Substantial losses occurred around pH 8.0 but it is not surprising that the reactions were less than quantitative in view of the fact that the starting material and products are aliphatic aldehydes. Nevertheless, it was important to establish which of the components of these reactions were decomposing because it would skew interpretation of the results if the decomposition selectively involved only one of the products.

In control experiments, **13** and **21** were shown to be stable under the conditions used for the reactions and workup. On the other hand, compound **4** was found to undergo self-condensation; chromatograms showed gradual formation of a broad new peak, t_R 14 min. The ESI mass spectrum gave a molecular ion at m/z 387 for which the first fragment ion involved loss of water to give m/z 369. The m/z 387 ion is consistent with the molecular ion ($M+H^+$) for the aldol dimer of **4** in which both deoxyribose moieties have been lost. A series of experiments carried out with different concentrations of **4** at pH 8.0 showed that formation of the t_R 14 min product was concentration dependent, further consistent with a dimerization reaction. Dimerization of **4** is unlikely to be of biological significance even at the highest levels of vinyl monomers to which an individual might be exposed.

Analogous studies were carried out with chloride salt **4**, formed by the reaction of chlorooxirane with dGuo. After HPLC purification, the partitioning of the salt of **4** between deglycosylation and FAPy formation was evaluated over pH values from 7.0 to 10.0 and

gave results that were essentially the same as those obtained with **4** prepared from acetoxoxirane.

The reaction of epoxyaldehydes with dGuo are known to occur mainly at the N² position leading to cyclic compounds that fragment to give etheno derivatives (16, 17). However, it was important to ascertain whether any of the N7 adduct was being formed which might degrade to FAPy adduct **12** and carbinolamine **22**. dGuo was treated with 2,3-epoxybutenal under conditions modeled on those that had been used for the reactions of acetoxo- and chlorooxirane. The crude reaction mixtures were worked-up by both acid and base hydrolysis; no attempt was made to search for the N7 adduct itself. Acid-catalyzed depurination failed to give detectable 7-oxoethyl-Gua (**13**); base treatment did not give FAPy **12** or products derived from **12**. The formation of **12** was evaluated by acid treatment of the base hydrolysates followed by air oxidation, which would have given **21** if **12** had been present. Thus, it can be concluded that N7 adduct **4** and the products of deglycosylation and ring-opening of **4**, are unique to reactions of the epoxides of vinyl monomers.

Discussion

Seminal studies of the reaction of chlorooxirane with deox-yguanosine and DNA were made by Scherer et al. (47) and Barbin et al. (7) who found that reaction occurs almost exclusively at the N7 position of dGuo. They measured the extent of formation of adduct **4** by deglycosylation to give 7-oxoethyl-Gua which represented ~98% of the products identified, the remainder being mainly the 3,N⁴-etheno derivative of dCyd. Later studies have also found small quantities of the etheno derivatives of dAdo and dGuo but the total of all etheno derivatives is no more than 2% of the product mixture (6).

We have now shown that cationic adduct **4** is sufficiently stable that NMR spectra and other characterization can be obtained if precautions are taken to avoid acid-catalyzed deglycosylation, base-catalyzed FAPy formation, and concentration-dependent self-condensation. The NMR spectrum of **4** is consistent with it existing mainly as the geminal diol. Scherer had previously reported the ¹H NMR spectrum of the protonated form of 7-oxoethyl-Gua (**13**) obtained in 2 M DCl in D₂O (47). Their spectrum of **13** closely resembled the spectrum of **4** including equivalence of the methylene protons although they assigned the structure as the cyclic hemiacetal, i.e., deglyco-sylated **4b**, rather than the geminal diol, on the basis of lability of the 2,4-dinitrophenylhydrazone and loss of water in field desorption mass spectra.

Our present studies reveal that **4** does not undergo exclusive deglycosylation at physiological pH. A competition exists between the deglycosylation reaction and opening of the imidazolium ring. The two reactions are reciprocally dependent on pH; at pH 7.4 the major product is indeed **13** but **12** is being formed ~10% of the time. We have found that FAPy base **19** is unstable, quickly forming carbinolamine base **14**; FAPy nucleoside **12** will form carbinolamine nucleoside **22**. The question becomes whether **22** could be the dominant source of pro-carcinogenic mutations in angiosarcomas induced by vinyl chloride rather than the generally accepted etheno derivatives.

Barbin et al. carried out a detailed study of the question of the relative miscoding potential of N7 adduct **4** and apurinic sites derived from it versus 3,N⁴-etheno-dCyd (7). The study was carried out *in vitro* by observing bypass of adducts formed by the reaction of chlorooxirane with poly(dG-dC); *E. coli* DNA pol I was used for this study. The rate of replication was significantly depressed; when 1.4% of the Gua residues had reacted, the rate of dGTP incorporation had been reduced by 73%. They concluded that misincorporation occurred mainly opposite dCyd and that **4** would miscode only 0.4-1.2% of the time while

3,*N*⁴-etheno-dCyd is a strongly mutagenic lesion. Due to the fact that dGuo adducts vastly outnumbered dCyd adducts, the ratio of misincorporation events opposite the two bases was 23:77. They estimated that one-half of the apparent mutagenic events opposite Gua were actually due to apurinic sites. Their final conclusion was that **4** is not miscoding.

Since the time of the Barbin studies, which were reported in 1985, much has been learned about the polymerases involved in DNA replication and techniques for studying replication have been greatly expanded. It is now clear that pol III is the main replicative DNA polymerase in prokaryotes. However, *E. coli* pol I is still a widely used model for a high fidelity, prokaryotic DNA polymerase due to its availability. Most DNA adducts are strong blocks to replication by replicative polymerases; alternative polymerases have been identified that bypass lesions but with reduced efficiency and fidelity. These are the SOS inducible polymerases (pols II, IV, and V) in prokaryotes and the Y-family polymerases η , ι , κ and rev 1 in mammals (49, 50); pols IV and V are also part of the Y-family. The FAPy adducts derived from oxidative damage to dGuo and methyl-FAPy (derived from initial N7-methylation) are known to be strong blocks to replication in *E. coli* (51–53). Thus, it is likely that any FAPy adduct **12** generated in the Barbin study would have blocked *E. coli* pol I and be interpreted as an abasic site. In addition, when other FAPy adducts are bypassed, either *in vivo* or *in vitro*, the correct insertion of dCTP opposite the lesion is largely observed in bacterial systems. However, the FAPy-adduct derived from oxidative damage to dGuo is more miscoding when replicated *in vivo* by mammalian cells (COS-7) (54).

In vitro studies with human lesion bypass DNA polymerases and site-specific mutagenesis studies in mammalian cells are needed to determine the miscoding potential of lesions derived from reactions of chlorooxirane with DNA. Current technology for carrying out such studies involves use of oligonucleotides containing single lesions with the structures and locations of these lesions rigorously defined (55). Our synthesis of carbinolamine **14**, specifically the preparation of allyl-FAPy nucleoside **16**, lays the groundwork for synthesis of the phosphoramidite reagent needed to assemble structurally defined oligonucleotides containing **22** needed for both *in vitro* and *in vivo* studies. Studies along these lines are ongoing in our laboratory.

An important issue is whether there is reason to expect that the FAPy adduct derived from chlorooxirane would be significantly mutagenic. The unsubstituted FAPy lesion formed by oxidation of dGuo is only minimally mutagenic in bacteria (56). Likewise, the FAPy adduct derived from 7-methyl-dGuo, although strongly blocking, is only minimally miscoding *in vitro* (51). However, the more complex FAPy lesion derived from aflatoxin B₁ is strongly mutagenic in *E. coli* (19).

A final issue is whether carbinolamine **22** would be persistent in the DNA of living cells. It is known that 7-oxoethyl-dGuo rapidly disappears from living cells whereas the etheno adducts are highly persistent (57) but the rates of repair of the oxoethyl-FAPy adduct and **22** are unknown. It should be noted that the N7 adduct of aflatoxin also rapidly disappears from cells while the corresponding FAPy lesion is highly persistent (58). The FAPy derived from dGuo oxidation and methyl-FAPy lesions are removed from DNA by base excision repair proteins (32, 59, 60) but the aflatoxin B₁-FAPy is excised by the nucleotide excision repair system in bacteria (61). The mechanism of repair of FAPy lesions of intermediate bulk and functionality, such as **22**, is unknown.

In summary, we have found that N7 adduct **4** formed by the reaction of the epoxides of vinyl monomers with dGuo undergoes ring opening to give significant quantities of the FAPy lesion at physiological pH. This adduct readily cyclizes to form the carbinolamine. This carbinolamine is formed uniquely by chlorooxirane and its analogs, whereas the etheno

lesions are also formed endogenously from epoxides of enals. Further work will be required to show that the lesion is formed and mutagenic in cells exposed to chlorooxirane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
HPLC chromatography of **14** using reverse phase (A) and normal phase (B) separation.

Scheme 1.
Reaction of Chlorooxirane with Deoxynucleosides

Scheme 2a.

^a (a) allyl bromide; (b) 1 M NaOH, 1 h; (c) 1 M HCl, 2 h, 60 °C; (d) OsO₄, NMO; (e) NaIO₄, pH 7.0; (f) 0.1 M HCl, 24 h, 60 °C; (g) O₂.

Scheme 3.

Scheme 4.
Proposed Fragment Ions Derived from Compound 14

Scheme 5.

Scheme 6.
Reaction Sequence Employed for the Analysis of 12

Table 1

Partitioning (24 h Reaction Time, 37 °C) of Cation 4 between Depurination to Give 13 and Ring Opening to Form 14 with Subsequent Transformation of 14 to 2-Aminopteridone (21) for Analysis

pH	<u>21/(13 + 21)</u>	<u>(13 + 21)/4</u>
	%	% yield
6.5	0	85
7.4	10	72
8.0	28	65
9.0	75	87
10.0	100	100